HHS Public Access

Author manuscript

Clin Chim Acta. Author manuscript; available in PMC 2016 February 05.

Published in final edited form as:

Clin Chim Acta. 2014 September 25; 436: 263–267. doi:10.1016/j.cca.2014.06.009.

Total testosterone quantitative measurement in serum by LC-MS/MS☆

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Abstract

Reliable measurement of total testosterone is essential for the diagnosis, treatment and prevention of a number of hormone-related diseases affecting adults and children. A mass spectrometric method for testosterone determination in human serum was carefully developed and thoroughly validated. Total testosterone from 100 μL serum is released from proteins with acidic buffer and isolated by two serial liquid–liquid extraction steps. The first extraction step isolates the lipid fractions from an acidic buffer solution using ethyl acetate and hexane. The organic phase is dried down and reconstituted in a basic buffer solution. The second extraction step removes the phospholipids and other components by hexane extraction. Liquid chromatography–isotopic dilution tandem mass spectrometry is used to quantify the total testosterone. The sample preparation is automatically conducted in a liquid-handling system with 96-deepwell plates. The method limit of detection is 9.71 pmol/L (0.280 ng/dL) and the method average percent bias is not significantly different from reference methods. The performance of this method has proven to be consistent with the method precision over a 2-year period ranging from 3.7 to 4.8% for quality control pools at the concentrations 0.527, 7.90 and 30.7 nmol/L (15.2, 228, and 886 ng/dL), respectively. This method provides consistently high accuracy and excellent precision for testosterone determination in human serum across all clinical relevant concentrations.

Keywords

Testosterone; Steroids; Hormone; LC-MS/MS

1. Introduction

Concentrations of testosterone in serum aid in the diagnosis and treatment of diseases such as hypogonadism [1,2], polycystic ovary syndrome [3], androgen deficiency in men [4–7], precocious or delayed puberty [8–10], and certain cancers [11–14]. Research findings describe associations of testosterone concentrations in blood with diabetes [15, 16], osteoporosis [17], cardiovascular diseases [15,18] and increased mortality [19,20]. Such

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associations have stimulated additional research studies and resulted in new clinical practice applications.

A common element to all these clinical and research applications is the need for reliable and accurate testosterone measurements. Considerable inaccuracy of testosterone assays has been described, especially at testosterone concentrations commonly observed in women and children [10,21–25]. Mass spectrometric methods were found to show improved accuracy and lower variability than immunoassays, especially at low testosterone concentrations [21,22,24]. This can be attributed in part to the higher specificity of this assay technology which is achieved by isolating the analyte from the sample matrix prior to analysis and by mass and structure-specific detection [26]. However, studies comparing routine clinical mass spectrometric assays against a reference measurement procedure showed inter-method variability in precision and accuracy [25]. Though less pronounced than the variability observed with immunoassays, the performance of many mass spectrometric assays remains inadequate for testosterone determination in all clinical and research settings. This created the need for developing new mass spectrometry-based methods for routine testing of testosterone that are suitable for measuring testosterone in men, women and children with appropriate accuracy, precision and throughput. To meet these needs, we developed a mass spectrometry-based method for the measurement of testosterone at all concentration levels in the general population.

2. Material and methods

2.1. Materials and chemicals

Testosterone traceable to Australian National Measurement Institute reference material (NMI M914) was obtained from Cerilliant (Round Rock, TX) and [2,3,4-$^{13}C_3$]-testosterone ($^{13}C_3$-testosterone) from IsoSciences (King of Prussia, PA). Ethyl acetate, hexane, 0.1% formic acid in acetonitrile, 0.1% formic acid in water, sodium acetate, and ammonium carbonate, glacial acetic acid, and ammonium hydroxide were acquired from Fisher Scientific (Suwannee, GA) and ethanol from Sigma-Aldrich (St. Louis, MO). All solvents were HPLC grade and chemicals were reagent grade. Steroids used for interference testing were obtained from Steraloids (Newport, RI), Sigma-Aldrich and Cerilliant and prepared at the concentration 6.93 nmol/L (200 ng/dL) in a solvent solution (water/acetonitrile/formic acid, 90:10:0.1, v/v/v).

Frozen human serum samples and charcoal processed (one time or six times) sera were purchased from Bioreclamation (Hicksville, NY). Three levels of quality control (QC) materials were prepared by pooling units of human sera from healthy donors (QC low: 0.527 nmol/L or 15.2 ng/dL, QC medium: 7.90 nmol/L or 228 ng/dL, QC high: 30.7 nmol/L or 886 ng/dL).

Commercially prepared, de-identified single donor human serum samples were obtained from several blood banks in the U.S. and serum specimens from a large study were used to evaluate the method. Study participants provided informed consent. The study protocol was approved by the CDC Research Ethics Review Board.

2.2. Sample preparation

Serum samples were thawed, mixed at room temperature and processed together with quality control samples, reagent blank, and calibrators. Calibrators were prepared in ethanol from 1 mg/mL certified testosterone solution with no more than 3 serial dilutions. Thirteen calibrator levels covering a range of 0.0347–34.7 nmol/L (1.00–1000 ng/dL) were processed together with each set of samples, quality control samples, and reagent blank. Samples and reagents were pipetted in 96-well plates (2 mL well volume, Seahorse Labware, Chicopee, MA) using a Hamilton Microlab STARLet Liquid Handler instrument (Reno, NV). The well plates were sealed using a PTFE coated sealing mat (ArctiSeal, Arctic White LLC, Bethlehem, PA). Serum samples (100 μL) and internal standard solution (IS, $^{13}C_3$-testosterone, 100 μL, 3.43 nmol/L or 100 ng/dL in ethanol) were combined and mixed for 30 min at room temperature. Buffer solution (100 μL, 0.5 mol/L sodium acetate, pH 5.5) was added, and the sample solution was mixed for 2 h. Liquid–liquid extraction (400 μL, ethyl acetate: hexane, 60:40, v/v) was performed twice. The solvents of the combined organic layers were evaporated using a Genevac EZ-2 evaporation system (Valley Cottage, NY) and the sample extract was re-dissolved in buffer solution (150 μL, 0.2 mol/L ammonium carbonate, pH 9.8). The sample solution was extracted twice using hexane (300 μL each) and the combined organic layers were evaporated. The sample extract was reconstituted (150 μL, 0.1% formic acid in water: 0.1% formic acid in Acetonitrile, 80:20, v/v) for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.

2.3. LC-MS/MS

Electrospray ionization LC-MS/MS analysis was carried out using an AB Sciex 5500 triple quadrupole mass spectrometry instrument (Foster City, CA) with a Shimadzu LC-10 AD VP HPLC system (Columbia, MD). Chromatographic separation was achieved at 40 °C using a reversed phase column (C18 Hypersil Gold, 50 × 3 mm, 3 μm, Fisher Scientific Suwanee, GA) protected by a guard column (10 × 3 mm, 3 μm) with the same material. The analyte was eluted from the column using a gradient with the eluent changing from 10% to 70% acetonitrile in water within 12 min. The column was washed for 3 min in 95% acetonitrile in water and equilibrated for 3 min at the initial eluent composition. Both solvents contained 0.1% formic acid. The flow rate and injection volume were 500 μL/min and 40 μL, respectively. Total testosterone was detected by selected reaction monitoring (SRM) in the positive ion mode, quantified by the ion transition *m/z* 289 → 97 for testosterone and 292 → 100 for IS. For confirmation of the analytes, the transitions *m/z* 289 → 109 for testosterone and 292 → 112 for IS were used.

The ionspray voltage and source temperature were 4.75 kV and 650 °C, respectively. Declustering, entrance and exit potentials were 96 V, 8 V and 12 V, respectively. Curtain gas, ion source gas 1, ion source gas 2, and collision gas were set to 45 psi, 35 psi, 55 psi, and 10 psi, respectively. The collision energies for quantitation ions of both testosterone and IS were 27 eV, and confirmation ions 31 eV.

2.4. Data analysis

The LC-MS/MS raw data were processed using Analyst software (version 1.5.1 or higher, AB Sciex, Foster City, CA). SAS (version 9.2) was used to define quality control limits and

to evaluate analytical runs against these limits using a multi-rule quality control approach [27]. The limit of detection was determined by using an AB Sciex 5500 triple quadruple instrument according to the procedures previously described [28].

We used the average sum of squared residuals (ASSR) and the average relative sum of squared residuals (RASSR) from 20 sets of calibration runs to choose the best fitting regression model from among linear and polynomial models with no weighting, weights of $1/X$, $1/X^2$, or 1/(Variance of Y). The ASSR was calculated by summing up the squared differences between the model predicted and observed Y values. The RASSR was calculated by dividing the ASSR by the average of the Y values. A linear calibration curve using a weight of 1/X was selected because this model had the smallest ASSR among all linear models and the second smallest RASSR among all models. Two quadratic models had slightly smaller ASSR values than the model chosen, but their RASSR values were larger than the RASSR values of the model chosen.

The measurement accuracy was calculated by analyzing 172 sera with reference values assigned by reference methods operated at CDC with the Joint Committee for Traceability in Laboratory Medicine (JCTLM) code C8RMP6 [29,30], NIST with the JCTLM code C4RMP2 [31] and the University of Ghent with the JCTLM code NRMeth-7 [32] as part of the CDC Hormone Standardization Program (Host) [29,33]. The measurement bias was assessed following CLSI protocol EP9-A2 [34] using a mean bias of ±6.4% against the reference method and a total error of ±16.7% for individual measurements as criteria [30,35]. Serum-based reference material SRM 971 at 2 concentrations was obtained from NIST, and ERM-DA345 and ERM-DA346 from LGC Standards for accuracy assessment. Correlation of our method with the reference methods using Deming regression and bias plot analysis was performed with Analyse-it (Analyse-it Software, Ltd., Leeds, United Kingdom, version 2.26).

Repeatability and method precision for low, medium and high QC pools were determined following CLSI protocol EP5-A2 [36] on 71 different days (two results on each of two runs per day) over a period of 2 years, using 3 calibrator lots and multiple operators.

Sample matrix effects (ME) were evaluated on 6 different matrices including ethanol, saline, one time charcoal processed serum, six times charcoal processed serum, male serum and female serum as previously described [30,37]. The ME was assessed using the following equation, $ME\% = B/A \times 100$, where B refers to the area count ratios of testosterone to $^{13}C_3$-testosterone obtained from samples in matrix and A in matrix free solution.

The extraction efficiency was assessed in triplicate using medium QC pools and adding the IS solution before sample preparation (A), and by adding the IS solution at the end of the sample preparation (B), before injecting the sample in the LC-MS/MS analysis. The efficiency was calculated using the following equation: measured value (B)/measured value (A) × 100.

Specificity was determined with two different approaches. In the first approach, potentially interfering compounds such as structural steroid analogs were added to samples to assess co-elution, while in the second approach quantitation ion/confirmation ion (QI/CI) ratios

obtained with patient samples were compared against those obtained with calibrators for each analytical run. The testosterone QI/CI ratios of calibrators and of 56 serum samples from the same batch of the above accuracy testing experiment were compared. Co-elution was assumed when the QI/CI ratio differed more than 20% [30,38,39].

Method ruggedness was assessed by comparing measurement values of the medium QC obtained with the described method against those obtained after modifying 5 main sample preparation parameters. The first parameter (**P1**) is the equilibrium time, during which sera are incubated with IS to achieve equilibrium between free and protein-bound IS (incubation durations tested: 20, 30 and 40 min). The second parameter is the pH of the dissociation buffer (**P2**), which is used to release testosterone from sex hormone binding protein and other proteins (pH levels tested: pH 5.2, 5.5 and 5.8). The third parameter is the time allowed for removing testosterone from proteins (**P3**, incubation times tested: 90, 120 and 150 min). The fourth parameter is the buffer concentration used to remove testosterone form proteins (**P4**, buffer concentrations tested: 0.3, 0.5 and 0.7 mol/L). The last parameter (**P5**) is the pH of buffer used in the second extraction step to remove polar lipids such as phospholipids (pH levels tested: pH 9.4, 9.8 and 10.2).

3. Results and discussion

The aim of this study was the development of an analytical method for measuring total testosterone in sera from men, women and children. Thus, this method needs to be able to measure this analyte over a wide concentration range starting at very low concentrations typically observed in children. The limits of detection (LOD) and quantitation (LOQ) [28] were determined to be 9.71 pmol/L (0.280 ng/dL) and 32.9 pmol/L (0.950 ng/dL), respectively. The linearity of this method was verified for the concentration range from 17.3 pmol/L (0.500 ng/dL) to 45.1 nmol/L (1300 ng/dL). To assure accurate and consistent calibration over such a wide concentration range, we use 13 calibrator levels and apply a weighted linear regression model. The sensitivity and measurement range of our method is sufficient for measuring testosterone levels in the general population. The signal-to-noise ratio ranged from 4.7 to 11.7 for 20 donor samples with testosterone concentration from 50 pmol/L to 140 pmol/L (0.40 to 4.10 ng/dL).

There was no undetectable testosterone concentrations in 250 individual donor samples from men and women age 6 years and older with serum testosterone concentrations ranging from 0.03 nmol/L (0.920 ng/dL) to 35.49 nmol/L (1024 ng/dL) (Table 1). A representative LC-MS/MS selected ion chromatograms of female samples were shown on Fig. 1.

The lack of sufficient accuracy and reliability of testosterone measurements is a major concern of the clinical and public health communities [40,41]. The Centers for Disease Control and Prevention is addressing this concern through its Hormone Standardization Program that evaluates assay accuracy using predefined performance criteria [35]. Using 172 serum samples with target values assigned by the National Institute for Standards and Technology, the University of Ghent and the CDC reference method covering a concentration range from 0.145 nmol/L (4.19 ng/dL) to 34.9 nmol/L (1007 ng/dL) showed no significant difference between our method and established reference methods with

Deming regression (intercept: −0.01, 95% CI: −0.05–0.04 nmol/L, slope: 0.99, 95% CI: 0.98–1.00, Fig. 2a) and with plot analysis (mean bias: −1.2%, 95%CI: −5.9–4.1%, Fig. 2b). Individual measurements on all 172 samples met the 16.7% total error criterion. The differences between target values of certified serum-based reference materials from NIST and LGC Standards are in average −1.3% (Table 2), which is within ±6.4% criterion for routine, clinical methods [30,35]. Furthermore, the mean bias observed with our method is smaller than those reported for other methods [25,42], especially at testosterone concentrations typically observed in women and children.

The consistency of measured values obtained after changing 5 sample preparation parameters suggests that this method is minimally affected by small variations in sample processing parameters (Fig. 3). This is reflected in part by the high long-term precision of this method. The method precision over 2 years covering 71 different days was 4.8%, 3.7%, and 3.8% for QC low, QC medium and QC high, respectively. The repeatability (within-run precision) was 3.7, 2.2, and 2.0% respectively. The method precision is well below the suggested maximum impression for total testosterone measurements of 5.3% [30,35] and smaller than the precision reported for other mass spectrometry methods [25,42].

Method sensitivity and precision can be affected by matrix effects that can cause ion suppression among other effects. The mean ME% determined in 6 different matrices is 100.2% (95% CI: 98.7–101.8%) as studied previously [30]. The matrices include ethanol, saline, 1 time charcoal processed serum, 6 times charcoal processed serum, male serum and female serum [30]. These findings suggest that measurements are minimally affected by the sample matrix, which might explain in part the high accuracy and precision obtained with this method. This method contains two liquid–liquid extraction steps. In the first step, serum lipids are separated from polar components. In the second step, polar lipids such as phospholipids are largely removed from the sample extract as evidenced in reduction in SRM (*m/z* 184–184) and precursor-ion scanning (pre *m/z* 184) signals that are indicative for these types of lipids [43,44]. Polar lipids typically accumulate on the column, deteriorate the separation and increase the ion suppression. A second extraction to remove polar lipids improved levels of precision (especially at low testosterone concentrations) and consistency of the chromatographic separation, and it prolonged column lifetime by about 5 times. In addition, we do not use protein precipitation, which is another common sample preparation approach [45]. Because testosterone is mostly bound to serum proteins, protein precipitation without complete dissociation of testosterone from binding-proteins and incomplete equilibrium of the IS with the binding proteins may result in incomplete or inconsistent analyte recovery [33]. Therefore, omitting protein precipitation seems to partially contribute to the very low measurement precision observed over 2 years. Despite multiple extraction steps, the extraction efficiency is 73% (95% CI: 70–76%). This is sufficient for quantitative measurements of testosterone at low concentrations such as those observed on women and children.

Measurement specificity has been another concern with testosterone measurements. To assess for potential interferences with known and unknown compounds we applied two approaches for interfering testing. In the first approach, the 28 structural analogs we tested showed no interfering peak in the chromatograms for the quantitation and confirmation ions

(Table 3). The second approach was used to assess interferences from unknown compounds. The average QI/CI ratio calibrators was 0.98 (95% CI: 0.82–1.15) while the ratio in 56 serum samples was in average 1.12 (95% CI: 1.08–1.15). The highest QI/CI ratio observed in an individual sample was 1.24, which is well-below the suggested maximum differences of 20% difference [30,38,39]. These findings suggest that this method is either minimally affected by interfering substances or that it is not at all affected. Interferences from components in serum separator tubes (SST) have been reported [46] but were not found with our method using BD Vacutainer® SST™ (Franklin Lakes, NJ) probably because of the use of different chromatographic conditions.

Some methods described in literature use derivatization procedures to enable analysis by GC-MS or to achieve higher sensitivity of LC-MS measurements [47–49]. Our method does not require derivatization, which minimizes complexity and facilitates automation. Furthermore, other methods employ ethers, such as methyl tertiary-butyl ether [24], diethyl ether [50], and diethyl ether/ethyl acetate mixtures [51] for the liquid–liquid extraction. However, these solvents are difficult to handle with automatic pipetting system and require special safety considerations. The solvents used in our method showed compatibility with automated liquid handlers and require fewer safety considerations than ethers [52–54].

The present method proofed a consistent result over a 2-year period at all concentration levels, as well as experimental variations with very minimal matrix effect and no or negligible difference for testosterone in neat and matrix. Measurement values obtained with this method are in high agreement with established reference methods [30] and meets performance criteria for accuracy used by the CDC Hormone Standardization Program [35]. This described method provides a consistently high accurate and sensitive LC-MS/MS assay for testosterone measurements in serum at all concentration levels typically observed in men as well as in women and children.

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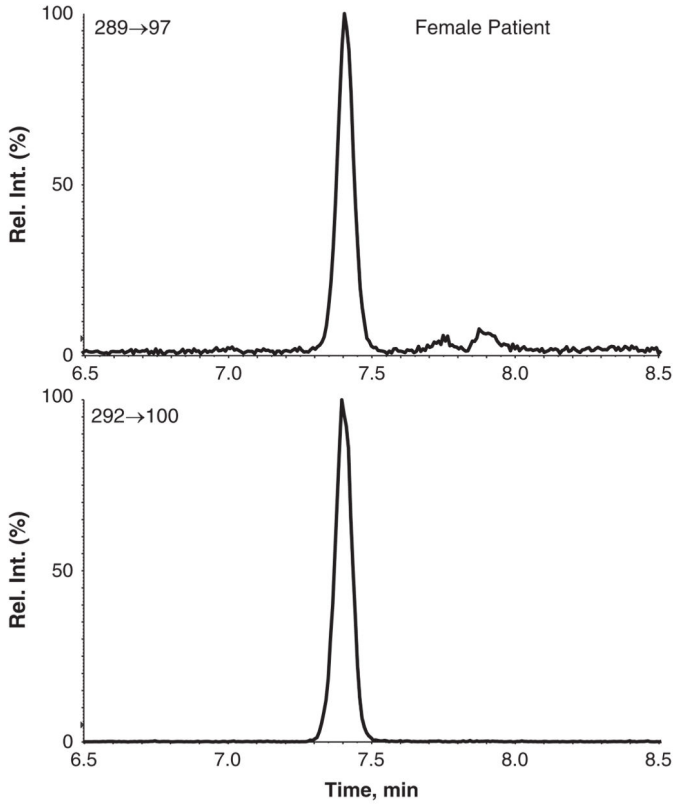

Fig. 1.

LC-MS/MS selected ion chromatograms of the quantification transitions for testosterone (top) and IS (bottom) in a female patient serum sample (1.00 nmol/L, 29 ng/dL).

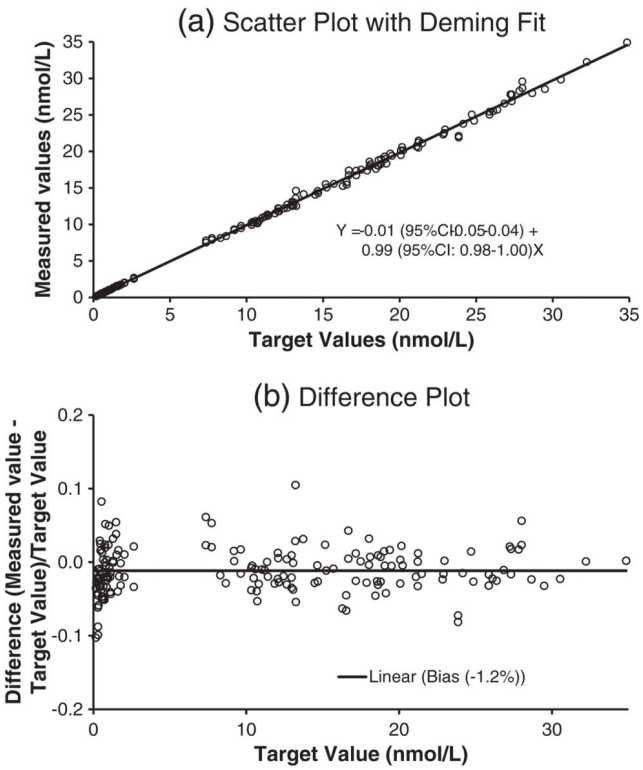

Fig. 2.

Deming regression (a) and bias plot (b) between described method and an established reference method using 172 individual health patient serum sample.

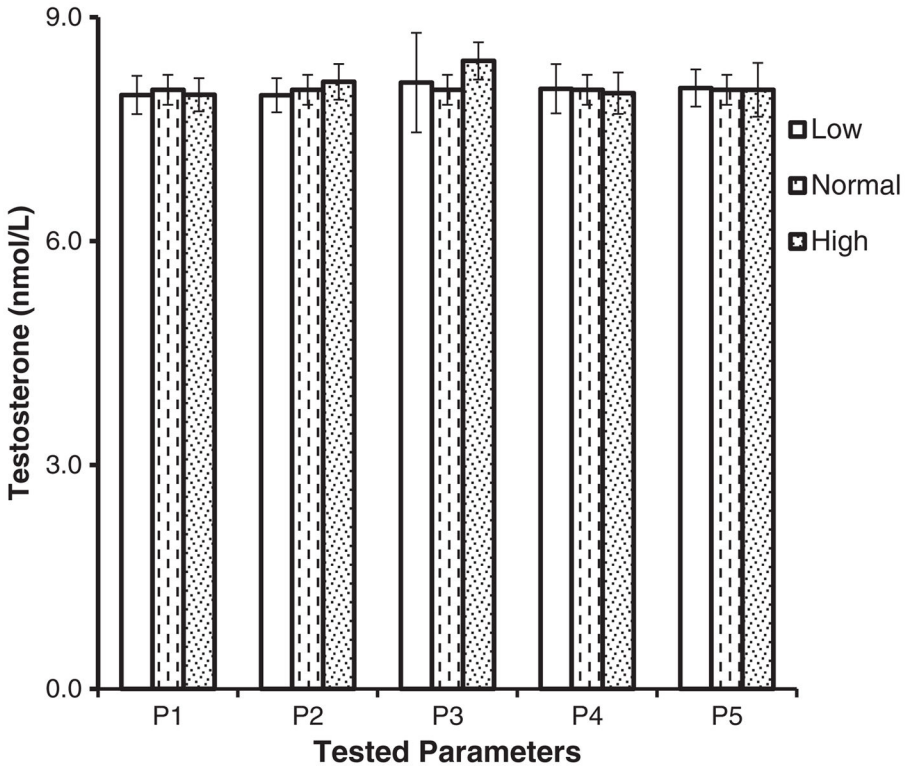

Fig. 3.

Ruggedness testing (n = 7; error bar: standard deviation). *Five main method parameters — P1: equilibration time of serum and internal standard (20, 30, and 40 min); P2: pH of dissociation buffer (5.2, 5.5, 5.8); P3: dissociation time of testosterone from binding proteins (90,120, and 150 min), P4: concentration of dissociation buffer (0.3, 0.5 and 0.7 mol/L); and P5: pH of deprotonation buffer (9.4, 9.8 and 10.2).

Table 1

Serum testosterone concentrations in individuals at difference age groups and sex.

Age group	Gender	Testosteronemedian (range)
6–11 years	Female (n = 50)	0.18 (0.03–0.91) nmol/L5.20 (0.92–26.14) ng/dL
	Male (n = 50)	0.17 (0.06–10.77) nmol/L4.94 (1.75–310.63) ng/dL
18 years and older	Female (n = 75)	0.80 (0.08–3.33) nmol/L23.02 (2.36–95.91) ng/dL
	Male (n = 75)	18.06 (1.10–35.49) nmol/L520.93 (31.66–1023.74) ng/dL

Table 2

Accuracy traceability to JCTLM listed materials and NIST serum reference materials.

Samples	Certified value	Measured value (N = 3)	%Bias
ERM DA345	19.7 nmol/L 570 ng/dL	19.4 nmol/L (95% CI: 18.4–20.5) 560 ng/dL (95% CI: 530–590)	−1.8
ERM DA346	0.890 nmol/L 25.5 ng/dL	0.881 nmol/L (95% CI: 0.849–0.912) 25.4 ng/dL (95% CI: 24.5–26.3)	−0.4
SRM 971 M	22.3 nmol/L 643 ng/dL	22.5 nmol/L (95% CI: 21.9–23.0) 649 ng/dL (95% CI: 633–664)	0.9
SRM 971 F	0.961 nmol/L 27.8 ng/dL	0.926 nmol/L (95% CI: 0.739–1.11) 26.7 ng/dL (95% CI: 21.3–32.1)	−4.0

Table 3

List of steroid hormones used in the interference analysis.

Steroids	Formula	Molecular weight	Supplier
Estrone	$C_{18}H_{22}O_2$	270.4	Cerilliant
Estradiol	$C_{18}H_{24}O_2$	272.4	Cerilliant
5,16-Androstadien-3b-ol	$C_{19}H_{28}O$	272.4	Steraloids
16, (5a)-Androsten-3-one	$C_{19}H_{28}O$	272.4	Steraloids
4,16-Androstadien-3b-ol	$C_{19}H_{28}O$	272.4	Steraloids
2,(5a)-Androsten-17-one	$C_{19}H_{28}O$	272.4	Steraloids
Estriol	$C_{18}H_{24}O_3$	288.4	Cerilliant
Dehydroepiandrasterone	$C_{19}H_{28}O_2$	288.4	Cerilliant
Epitestosterone	$C_{19}H_{28}O_2$	288.4	Sigma-Aldrich
Trans-dehydroandrosterone	$C_{19}H_{28}O_2$	288.4	Sigma-Aldrich
Trans-androsterone	$C_{19}H_{30}O_2$	290.4	Sigma-Aldrich
5-Androstenediol	$C_{19}H_{30}O_2$	290.4	Steraloids
Androsterone	$C_{19}H_{30}O_2$	290.4	Cerilliant
Etiocholan-3a-ol-17-one	$C_{19}H_{30}O_2$	290.4	Sigma-Aldrich
Dihydrotesosterone	$C_{19}H_{30}O_2$	290.4	Cerilliant
17a-Ethinylestradiol	$C_{20}H_{24}O_2$	296.4	Sigma-Aldrich
19-Norethindrone	$C_{20}H_{26}O_2$	298.4	Sigma-Aldrich
D(−)-Norgestrel	$C_{21}H_{28}O_2$	312.4	Sigma-Aldrich
Progesterone	$C_{21}H_{30}O_2$	314.5	Cerilliant
5-Pregnen-3β-ol-20-one	$C_{21}H_{32}O_2$	316.5	Steraloids
Pregnenolone	$C_{21}H_{32}O_2$	316.5	Sigma-Aldrich
17α-Hydroxyprogesterone	$C_{21}H_{30}O_3$	330.5	Cerilliant
17α-Hydroxypregnenolone	$C_{21}H_{32}O_3$	332.5	Steraloids
Corticosterone	$C_{21}H_{30}O_4$	346.5	Cerilliant
11-Deoxycortisol	$C_{21}H_{30}O_4$	346.5	Cerilliant
Cortisone	$C_{21}H_{28}O_5$	360.4	Steraloids
Hydrocortisone	$C_{21}H_{30}O_5$	362.5	Steraloids
Dehydroepiandrosterone sulfate	$C_{19}H_{28}O_5S$	368.5	Steraloids